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Physical exercise mitigates doxorubicin-induced brain cortex and cerebellum mitochondrial alterations and cellular quality control signaling

Marques-Aleixo I. [a,*], Santos-Alves E. [a], Balça M.M. [a], Moreira P.I. [b,c], Oliveira P.J. [d], Magalhães J. [a], Ascensão A. [a]

[a] CIAFEL — Research Centre in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto, Portugal
[b] CNC — Centre for Neuroscience and Cell Biology, University of Coimbra, Portugal
[c] Institute of Physiology, Faculty of Medicine, University of Coimbra, Portugal
[d] CNC — Center for Neuroscience and Cell Biology, University of Coimbra, UC Biotech Building, Biocant Park, Cantanhede, Portugal

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ABSTRACT

Doxorubicin (DOX) is a highly effective anti-neoplastic agent, whose clinical use is limited by a dose-dependent mitochondrial toxicity in non-target tissues, including the brain. Here we analyzed the effects of distinct exercise modalities (12-week endurance treadmill—TM or voluntary free-wheel activity—FW) performed before and during sub-chronic DOX treatment on brain cortex and cerebellum mitochondrial bioenergetics, oxidative stress, permeability transition pore (mPTP), and proteins involved in mitochondrial biogenesis, apoptosis and auto(mito)phagy.

Male Sprague–Dawley rats were divided into saline-sedentary (SAL + SED), DOX-sedentary (DOX + SED; 7-week DOX (2 mg·kg^{-1} *per* week)), DOX + TM and DOX + FW. Animal behavior and post-sacrifice mitochondrial function were assessed. Oxidative phosphorylation (OXPHOS) subunits, oxidative stress markers or related proteins (SIRT3, p66shc, UCP2, carbonyls, MDA, –SH, aconitase, Mn-SOD), as well as proteins involved in mitochondrial biogenesis (PGC1α and TFAM) were evaluated. Apoptotic signaling was followed through caspases 3, 8 and 9-like activities, Bax, Bcl2, CypD, ANT and cofilin expression. Mitochondrial dynamics (Mfn1, Mfn2, OPA1 and DRP1) and auto(mito)phagy (LC3II, Beclin1, Pink1, Parkin and p62)-related proteins were measured by semi-quantitative Western blotting.

DOX impaired behavioral performance, mitochondrial function, including lower resistance to mPTP and increased apoptotic signaling, decreased the content in OXPHOS complex subunits and increased oxidative stress in brain cortex and cerebellum. Molecular markers of mitochondrial biogenesis, dynamics and autophagy were also altered by DOX treatment in both brain subareas. Generally, TM and FW were able to mitigate DOX-related impairments in brain cortex and cerebellum mitochondrial activity, mPTP and apoptotic signaling.

We conclude that the alterations in mitochondrial biogenesis, dynamics and autophagy markers induced by exercise performed before and during treatment may contribute to the observed protective brain cortex and cerebellum mitochondrial phenotype, which is more resistant to oxidative damage and apoptotic signaling in sub-chronically DOX treated animals.

1. Introduction

Doxorubicin (DOX) is a highly effective antibiotic used to treat several types of malignancies. Despite its high efficacy, DOX clinical use is limited by dose-related severe adverse effects on non-target tissues, including the heart and the brain (Cardoso et al., 2008; Carvalho et al., 2009; Wallace, 2007). Although it is considered that DOX does not cross the blood–brain barrier (Bigotte and Olsson, 1982; Ohnishi et al., 1995), several studies in patients undergoing DOX-based chemotherapy reported persistent changes in cognitive function, commonly referred to as "chemo brain", sometimes lasting years after cessation of chemotherapy (Aluise et al., 2010).

Although the biochemical basis for DOX toxicity resulting in cognitive impairments requires further studies, it has been proposed that the chemotherapy-related neurotoxicity is mediated through cytokines as DOX increases the systemic production of tumor necrosis factor-α (TNFα) (Aluise et al., 2010; Tangpong et al., 2006), which can migrate into the brain and stimulate locally its production (Seruga et al., 2008). The elevation of these pro-inflammatory mediators and consequently brain pro-inflammatory environment caused by DOX may lead to oxidative stress, mitochondrial dysfunction, increased susceptibility to neuronal mitochondrial permeability transition pore (mPTP)

* Corresponding author at: Research Centre in Physical Activity Health and Leisure, Faculty of Sport, University of Porto, Rua Dr. Plácido Costa 91, 4200-450 Porto, Portugal.
E-mail address: inesmaleixo@hotmail.com (I. Marques-Aleixo).

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opening (Cardoso et al., 2008) and possibly activation of apoptotic signaling (Joshi et al., 2010; Pal et al., 2012; Tangpong et al., 2007, 2006). These mechanisms seem to be central to explain DOX-induced brain mitochondrial toxicity and may contribute to the deterioration of cognitive function often observed in patients undergoing chemotherapy (Joshi et al., 2010; Tangpong et al., 2006, 2007).

Physical exercise is a neuroprotective strategy to mitigate impairments on brain function as it inhibits oxidative stress and apoptotic signaling, resulting in beneficial adaptations and in a healthier neuronal phenotype. Together with increased mitochondrial biogenesis and oxidative phosphorylation (OXPHOS) activity, the data emphasize the role of mitochondria as plastic organelles central for the adaptations resulting from physical exercise (Marques-Aleixo et al., 2012). Recently, our group reported that treadmill and voluntary free wheel running improved behavioral performance, brain cortex and cerebellum mitochondrial fitness including OXPHOS capacity, and altered oxidative status, mPTP susceptibility, apoptotic signaling, mitochondrial dynamics and quality control signaling (Bernardi, 2013).

Although the effects of chronic physical exercise against DOX-induced impairments in the cardiovascular system, including deterioration in cardiac hemodynamics, mitochondrial bioenergetics and associated pathways have been previously investigated (Ascensao et al., 2007, 2011b, 2012), whether brain cortex and cerebellum mitochondrial alterations associated with sub-chronic DOX administration are limited by long-term physical exercise has yet to be determined. Our objective was to analyze the effects of two modalities of long-term exercise with distinct characteristics, treadmill and voluntary activity, performed before and during the course of sub-chronic DOX administration, on brain cortex and cerebellum mitochondrial respiratory activity, OXPHOS components and biogenesis, susceptibility to mPTP opening, apoptotic signaling, oxidative stress, as well as mitochondrial dynamics and auto(mito)phagy signaling. The integrated analysis of these mitochondrial-related alterations with behavioral performance may contribute to understand the mechanisms by which physical exercise is useful for improving cognitive function in patients undergoing chemotherapy with DOX.

2. Methods

2.1. Reagents

Deionized water (18.7 MΩ) from an arium® 611VF system (Sartorius, Göttingen, Deutschland) was used. Doxorubicin hydrochloride, commercial/clinic use, was obtained from *Ferrer Farma* (Barcelona, Spain), prepared in a sterile saline solution, NaCl 0.9% (pH 3.0, HCl) and stored at 4 °C for no longer than five days upon rehydration. Caspases 3, 8 and 9 substrates were purchased from Merck KGaA (Darmstadt, Germany), Commercial RANSOD kit from Randox Labs (Antrim, UK), chemiluminescent reagent ECL-Plus™ 104 (RPN2236) from GE Healthcare (Amersham BioSciences UK Ltd., Buckinghamshire, UK) and PVDF membranes (#IPVH00010) from Millipore (Billerica, MA, USA). Primary antibodies were purchased as follows: anti-OXPHOS (ab110413, a premixed cocktail containing 5 mouse antibodies: against CI subunit NDUFB8 (ab110242), CII-30 kDa (ab14714), CIII-Core protein 2 (ab14745) CIV subunit I (ab14705) and CV alpha subunit (ab14748)), anti-PGC1-α (ab106814), anti-cyclophilin D (ab110324), anti-p62 (ab56416), anti-OPA1 (ab119685), anti-PINK1 (ab23707) and anti-Parkin (ab15954) from *Abcam* (Cambridge, UK); anti-Bax (#2772), anti-Bcl-2 (#2870), anti-DRP1 (#8570), anti-cofilin (#5175), anti-SIRT3 (#2627), anti-p66sch (#2432) and anti-beclin1 (#3495) from *Cell Signaling Technology* (Danvers, MA, USA); anti-LC3 (PD014) from *MBL Medical & Biological Labs* (Nagano, Japan); anti-DNP (D9656) and anti-UCP2 (SAB2501087) from *Sigma Aldrich* (Barcelona, Spain); anti-shc/p66(pSer^{36}) (6E10) from *Calbiochem* (Merck Millipore Darmstadt, Germany); and anti-ANT (sc-9299), anti-Mfn1 (sc-50330), anti-Mfn2 (sc-50331), anti-TFAM (sc-23588), anti-TOM 20 (sc-11415) and anti-β-actin (sc-1616) from *Santa Cruz Biotechnology Inc.* (Santa Cruz, CA, USA). Secondary antibodies were purchased from *Santa Cruz Biotechnology Inc.* (Santa Cruz, CA, USA). All other chemicals were purchased from *Sigma Aldrich* (Barcelona, Spain).

2.2. Animals

All experimental procedures involving animals were performed in accordance with the European Convention for the Protection of Vertebrate Animal Used for Experimental and Other Scientific Purposes (CETS no. 123 of 18 March 1986 and 2005 revision) and the Commission Recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes [C (2007) 2525]. The authors are accredited by the Federation of Laboratory Animal Science Associations (FELASA) for animal experimentation (class c). The Ethics Committee of the Research Centre in Physical Activity, Health and Leisure (Faculty of Sport, University of Porto) approved the experimental protocol.

Twenty-four male Sprague–Dawley rats (aged 21 days old) were obtained from Charles River Laboratories (L'Arbresle, France) and were randomly divided into four groups (n = 6 *per* group): saline sedentary (SAL + SED), DOX sedentary (DOX + SED), DOX treadmill endurance training (DOX + TM) and DOX free wheel voluntary physical activity (DOX + FW). During the experimental protocol, animals were individually housed and were maintained in a room at normal atmosphere (21–22 °C; 50–60% humidity), receiving food *ad libitum* (Scientific Animal Food and Engineering, A04) and water *ad libitum* in 12-h light/dark cycles.

2.3. Endurance training and voluntary physical activity

The animals from the TM group were exercised 5 days/week (Monday–Friday) in the morning (between 10:00 and 12:00 AM), for 12 weeks on a LE8700 motor-driven treadmill (Panlab, Harvard, USA). The protocol included 5 days of habituation to the treadmill with 10 min of running at 15 m/min, with daily increases of 5–10 min until 60 min of running was achieved (end of week 0). Habituation was followed by 12 weeks of continuous running (60 min/day) and the velocity increased gradually from 18 m/min to 27 m/min on week 7 and then the velocity was gradually adjusted down to 20 m/min until the end of the protocol.

The animals from the FW group were housed in a polyethylene cage equipped with a running wheel (circumference = 1.05 m, Type 304 Stainless steel (Cat. No. 2154F0106-1284L0106) Tecniplast, Casale Litta, Italy). The rats were allowed to exercise *ad libitum* with an unlimited access to the running wheel 24 h/day. Running distance was recorded using ECO 701 Hengstler (Lancashire, UK).

2.4. Doxorubicin treatment

After the 5th week of endurance training or free wheel exercise, the animals were treated with a sub-chronic protocol of seven weekly injections with DOX or sterile saline solution NaCl 0.9% (intraperitoneal injection 2 mg/kg of body weight) (Serrano et al., 1999; Zhou et al., 2001a, b) (see Fig. 1). The animals assigned to the TM groups received DOX or SAL injections during the weekend in a day-off training.

2.5. Behavioral Y-maze test

The Y-maze spontaneous alternation paradigm is based on the natural tendency of rodents to explore a novel environment. When placed in the Y-maze, animals will explore the least recently visited arm, and thus tend to alternate visits between the three arms. For efficient alternation, animals need to use working memory and therefore, they should maintain an ongoing record of most recently visited arms, and continuously update such a record. An animal with an impaired working memory